Research article

Journal of RAMAN SPECTROSCOPY

Received: 30 June 2014 Revised: 2 February 2015 Accepted: 6 February 2015 Published online in Wiley Online Library: 8 March 2015

(wileyonlinelibrary.com) DOI 10.1002/jrs.4674

# Novel highly sensitive Cu-based SERS platforms for biosensing applications

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**Novel surface enhanced Raman spectroscopy (SERS) platforms have been prepared and used for the bacteria detection. Unlike typical, expensive SERS platforms prepared from gold or silver, the presented platforms are prepared using copper. A new, simple, cost-efficient and fast high pressure method is used for platform fabrication, through the decomposition of copper hydride. The platform enhancement factors are verified using the malachite green isothiocyanate as a standard. The platforms exhibit extremely high SERS enhancement factors depending on pressure used for their preparation. The calculated enhancement factors have been found in the range between $1.5 \times 10^6$ and $4.6 \times 10^7$. The SERS spectra reproducibility is established both across a single platform and among different platforms. The average spectral correlation coefficient ($\Gamma$) has been calculated to be 0.82. Fully characterized SERS platforms have then been used for detecting *Staphylococcus aureus* bacteria. These novel platforms have great potential to become excellent tools for biological or medical diagnostics as an alternative to more common silver or gold SERS platforms. Copyright © 2015 John Wiley & Sons, Ltd.**

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**Keywords:** copper-based surfaces; surface enhanced Raman spectroscopy; high pressure methods; enhancement factors

## Introduction

The surface enhanced Raman spectroscopy (SERS) offers a possibility to study Raman vibrations in very thin samples. Nanostructures, such as nanocolloidal metallic particles in solution or deposited on a solid substrate have shown extraordinary large (even as high as $10^{14}$–$10^{15}$) enhancement of Raman signal. In SERS, Raman signal increases because of the local field enhancement associated with resonant excitation of surface plasmons (a long-range classical electromagnetic effect) and chemical enhancement on the metal surface (a short-range chemical effect).[1] Current knowledge considers the first mechanism to be the major part in the observed intensity enhancement (~$10^4$–$10^7$), while the chemical effect contributes to a considerably lower enhancement (~10–$10^2$).[2]

The surface enhanced Raman spectroscopy has significant advantages over the fluorescence spectroscopy,[3] those include the ability to multiplex numerous analytes because of the narrow line widths (~10 nm) and molecular specificity of SERS spectra.[4] The SERS advantages have been demonstrated in the flexibility in the label choice, insensitivity to quenching by oxygen or other species and excellent sensitivity to target analyte.[5] One of the most promising features offered by SERS is the possibility to use the platforms in biosensing, however, prior to the routine application optimized, stable substrates are needed. More importantly, the desired substrates should exhibit uniformly high enhancements. Because the enhancement factor strongly depends on the substrate nanomorphology,[6] the precise control over substrate surface in the nanoscale is crucial for successful platform preparation. Many methods can be used to fabricate SERS platforms, including electrochemical metal deposition, electrochemical surface modification or deposition on a chosen template.[7] However, an optimal fabrication method should consider simultaneously the ability to produce nanostructured arrays with specific size, shape, alignment, and architecture within very tight tolerances.

Gold (Au) or silver (Ag) are usually chosen to be materials for SERS platform preparation, nevertheless, cost-efficient copper (Cu) is less common. Unstructured gold substrates typically give enhancement factor in the order of magnitude of $4.5 \times 10^6$, whereas gold colloid only ~$1 \times 10^5$,[6,8] which is opposite to silver systems, where large substrates give enhancement of $10^5$,[9] and silver systems sols even up to $10^7$–$10^{10}$.[10] In literature, one can find only few reports devoted to the study regarding SERS enhancement obtained for copper platforms. The enhancement obtained for colloidal copper-based systems and large substrates were reported to be up to $10^5$.[11] In response to the demand of producing a new platform with improved properties, the method of copper platform grafted on silicon wafers was elaborated.[12] The enhancement factor obtained for the copper platform on silicon was very high and equal to $2.29 \times 10^7$. Noticeably, the silver substrates were found to be very unstable over longer time, even if covered with a film of another metal.[13] In comparison to Ag-based platforms the Cu-based platforms are very promising because of improved physical stability.[14] Additionally the silver is known from its bactericide properties, which can cause undesired effects such as DNA denaturation;[15] thus, also in that sense, the copper is advantageous over silver.[16] There are only few techniques, which can be used for large Cu-based platform fabrication, i.e. electrochemical

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deposition with surface modification using oxidation–reduction cycles,[17] lithography,[18] and evaporation.[19] The colloidal systems are usually prepared using chemical reduction of a cupric salt with borohydride as the reduction agent.[20] All these methods are time-consuming 'wet' techniques and usually result in platforms with a non-uniform covering.

In SERS, the most common difficulties are associated with the unambiguous interpretation of the obtained results. The enhancement factors in relation to the surface roughness, the shape, and the size of either particles or the whole particle clusters can significantly influence readout signal. Such nanoscopic variations give different Raman intensities resulting in different enhancement factors. Thus, novel methods for SERS platforms preparation, giving an opportunity to control the size of metal particles onto the prepared surfaces are highly desirable. Considering all these particulars, in this paper we present, novel Cu-based SERS platforms prepared using newly developed high pressure method as competitive way to the already existing techniques. In this method, high pressure is used for the decomposition of the copper hydride (CuH).[21] Our goal is to obtain a SERS platform, which can be prepared in a simple, reliable, and high-throughput way useful for both fundamental studies and medical diagnostics. We expect that the early-detection platform fabricated in a simple and cost-efficient way, which gives SERS enhancement comparable to the silver platforms, will act as a reliable tool to assist clinicians to decide for appropriate treatment of patients. In order to demonstrate the usefulness of our SERS platform for recognition in biological samples, the detection of *Staphylococcus aureus* bacteria is also presented.

## Experimental section

### The preparation of Cu-based SERS platforms

Novel copper-based SERS platforms were prepared using a new high pressure method, in which the decomposition of copper hydride is induced via pressure.[22] At first, the starting compound (CuH) was synthesized using a method presented in the literature.[23] Briefly, 40 ml of 0.6 M copper sulfate ($CuSO_4$) was added under an inert atmosphere to 40 ml of 2.4 M hydrophosphorous acid ($H_3PO_2$). All reagents were mixed and the reaction was maintained at 45 °C. The obtained red-brown precipitate was cooled in dry ice with n-propanol, filtrated and washed with ice water, cold absolute ethyl alcohol, and diethyl eter. After that, the product was dried under vacuum and distributed into glass vials in an amount adequate to preparing one platform. The final product was characterized by X-ray diffraction technique (Fig. S1; Supporting Information). Further, the vials with copper hydride were inserted into dry ice and left in the fridge to establish the temperature around −78 °C, in which the pure, anhydrous copper hydride is stable.[24] To prepare SERS platform, the small quantity of CuH was inserted to the pressure cell and the pressure was applied using hydraulic press. In this way, the decomposition of CuH was fulfilled resulting in the copper platform in a form of a disk. Taking into account the endurance of the used pressure cell, for preparation of the copper platforms, the pressure from the range of 572–973 MPa was applied. Employed pressure, in all cases, turn out to be sufficient for the copper platforms fabrication. The details of the procedure are written in Table 1. Additionally, the pretreatment procedure for SERS substrate preparation just before usage, was employed. In that step, for chosen SERS platform (B and E), the glacial acetic acid was used for removing the copper oxide from the platform of copper without etching the copper surface.[25]

**Table 1.** Examples of platforms with applied pressure for its fabrication, the size of obtained copper crystals taken from Atomic force microscopy (AFM) and scanning electron microscope (SEM) measurements, and the enhancement factor calculated for the intensity of bands at 1175 $cm^{-1}$

| Platform | Used pressure (MPa) | Size of crystals (nm) from: | | $E_F$ |
|---|---|---|---|---|
| | | AFM | SEM | |
| A | 572 | 30–60 | 30–90 | $1.1 \times 10^7$ |
| B | 653 | 30–70 | 30–90 | $1.9 \times 10^{7a}$ |
| C | 663 | 30–60 | 30–60 | $1.5 \times 10^7$ |
| D | 713 | 30–60 | 30–60 | $6.3 \times 10^6$ |
| E | 908 | 30–80 | 30–90 | $4.6 \times 10^{7a}$ |
| F | 973 | 30–80 | 30–90 | $3.5 \times 10^6$ |

[a]The enhancement factors obtained for platform after treatment with acetic acid (below 30 s) before further preparation to the measurement.

### Standard and biological sample for SERS

The malachite green isothiocyanate (MGITC), chosen as a standard, was purchased from Sigma Co. (Sigma, St, Louis, MO, USA), and used without further purification. Water (resistivity over 18 Mu), purified using a Milli-Q plus 185 system (Millipore, Molsheim, France) was used throughout the process.

### Atomic force microscopy

Atomic force microscopy (AFM) images were taken with a MFP-3D Bio Asylum Research/Oxford Instruments, equipped with active vibration isolation table Accurion Halcyonics Micro and acoustic enclosures. AFM microscope was operating in AC-Mode (Tapping) using AC160TS-R3 tip-probe from (Olympus, Hachioji-shi, Tokyo, Japan) (material Al-coated silicon, probe radius = 9 nm, $k$ = 26 N/m, $f$ = 300 kHz). The surface coverage and the diameter of the obtained copper surfaces were evaluated directly from obtained images.

### Raman spectroscopy and surface enhanced Raman spectroscopy

Measurements were carried out on dried samples using a Renishaw inVia Raman system equipped with a 632.8 nm helium–neon (HeNe) laser. For bacteria detection diode 785 nm laser was used. The light from the laser was guided through a line filter and focused with a 50× microscope objective [Numerical Aperture (NA) = 0.25] on a sample mounted on an X–Y–Z translation stage. The Raman scattered light was collected by the same objective through a holographic notch filter to block out Rayleigh scattering. An 1800 groove/mm grating was used to provide a spectral resolution of 5 $cm^{-1}$. The beam diameter was approximately 2.5 μm with 5 μW laser power onto the sample. Spectra were taken around 20 times in different places for each platform.

### Bacteria culture

The *S. aureus* were cultivated in liquid Lysogeny broth (LB) growth medium, and then incubated in a shaker (150 rpm) at 30 °C for 24 h. Afterwards, the bacteria were dispersed in Millipore water and centrifuged for 10 min at 4000 rpm to avoid damage of the cell membrane. Finally, the supernatant liquid was discarded and the bacterial cells were redispersed in Millipore water. The centrifugation process was repeated 5 times to obtain a solution of clean

bacterial cells. The purified bacteria were dispersed in Millipore water to a concentration of $10^2$ CFU/ml. The density of bacterial cells was determined by counting the number of colonies grown on a Petri dish starting from a known amount of the medium. The count was taken after 1 day of cultivation at 37 °C. About 10 μl of aqueous bacterial solution was applied to the SERS platform and incubated at 30 °C for 1 h. The sample was washed with Millipore water and dried in a flow of argon gas before using for Raman measurements.

## Results and discussion

### The characterization of the copper-based platforms and their stability

The novel copper-based platforms for SERS measurement were prepared using high pressure method in which the pressure induced decomposition of copper hydride. It is known from previous work that the copper hydride decomposes spontaneously at ambient condition. Only pressures higher than 8400 MPa stabilize this material efficiently.[21] Below this pressure, different external pressure can affect decomposition kinetics. Thus, different sizes of obtained copper crystals are mainly due to the kinetic effects. As can be seen in Table 1, relationships exist between the different average sizes of obtained copper crystals and the applied pressure for substrate preparation. For example, for platforms A, B, C, and D, the obtained sizes of Cu crystals are in the range of 30–70 nm. The sizes of the copper crystals are growing with a higher pressure applied. Thus, in the case of platforms E and F (the pressure above 900 MPa), the copper crystals of 30–80 nm sizes were obtained. Noticeably, as can be seen on Fig. 1 (the AFM images of two D and E platforms fabricated at different pressures), the obtained structures are uniformly covering the whole area of platforms. The AFM and additionally scanning electron microscope (SEM) images of the all platforms are presented on Figs. S2 and S3 (Supporting Information). Moreover, we have developed a way to improve the properties and enhancing ability of the newly obtained platforms through applying the pretreatment procedure. If one takes into account the fact that copper almost immediately reacts with oxygen from the air forming native oxide layer, it becomes important to remove copper oxides from the platform surface prior to applying any analytes. Therefore, before dipping the platforms in analyte solution, the platforms were immersed in glacial acetic acid

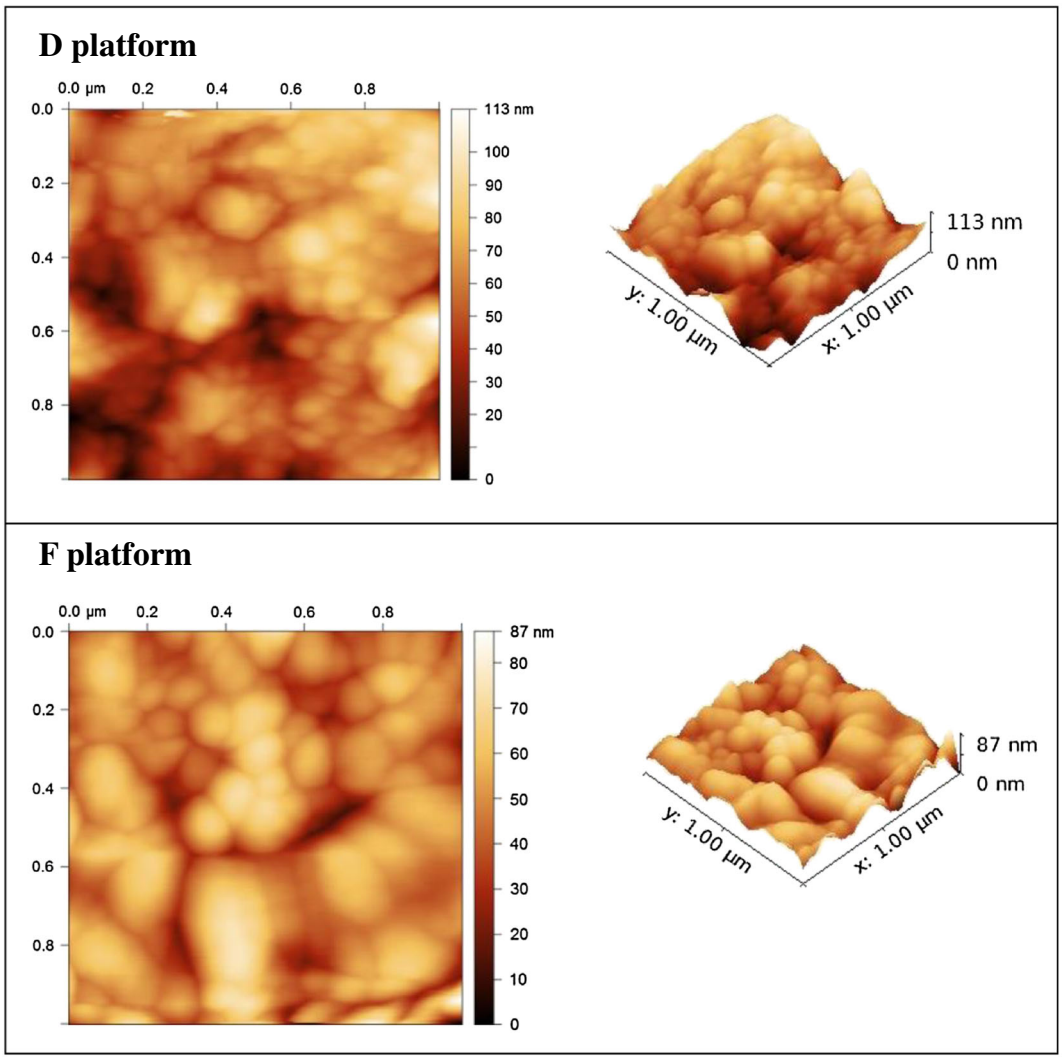


**Figure 1.** The two-dimensional and three-dimensional Atomic force microscopy images of chosen platforms obtained with different pressure; for exact condition see Table 1. (This figure is available in colour online at wileyonlinelibrary.com/journal/jrs.)

(platforms B and E) for 30 s and dried under a nitrogen gas flow. This procedure supports removal of copper oxides without etching the copper surface.[25]

In order to verify SERS activity for the obtained platforms, the enhancement factors were calculated using chosen standard MGITC. First, the Raman spectrum was recorded for 0.5 M MGITC solution. Second, the concentration of $10^{-6}$ M MGITC was prepared for SERS measurement. In normal Raman spectroscopy, no signal is usually observed at such low MGITC concentrations. To ensure the resonant condition for MGITC, for SERS measurements 633 nm laser excitation was chosen, where the maximum of absorption for these molecules exist.[26] Therefore, under such Raman experimental conditions for MGITC, we gathered the surface enhanced resonance Raman spectra (SERRS scattering). The copper-based platforms were left in the solution of $10^{-6}$ M MGITC for 3 h. After that time, SERS spectra were recorded (see Fig. 2). All observed vibrations are typical and originate from MGITC moieties and can be considered as a fingerprint of that molecule.[26–28]

For treated platforms B and E (see Table 1 for details), the enhancement factor was found to be in the order of $10^7$, while for untreated platforms (A and F in Table 1), the enhancement factor was two times to even one order of magnitude lower. That enhancing effect is related to the direct adsorption of analyte molecules on the copper surface. To put more stress onto the importance of the invented and employed procedure of SERS substrate preparation, the SERS spectra of MGITC molecules were measured for two platforms with and without treatment of glacial acetic acid (Fig. 2a). As one can see, the SERS signal intensity of MGITC is much higher (about two times) onto treated platform than the untreated SERS substrate. That is because of the fact that in a case of untreated platform a $CuO/Cu_2O$ exist on its surface and the MGITC can react with them, producing an insoluble salts of CuS and/or $Cu_2S$.[29] These species may block the active area of the SERS platform, which directly influence the number of MGITC molecules attached to the surface platform and further cause decreasing of the MGITC SERS signal intensity. Thus, using the treatment method will assure significantly better condition for SERS effect. On the other hand, fabricated SERS platform are very stable in time. Basically, the platform is stable directly after the preparation of a copper platform using high pressure method. We have checked the stability of obtained copper-based platform with MGITC as analyte in SERS experiment with one platform freshly prepared and the same sample stored at room temperature after 12 weeks. For that purpose, the platform C was chosen. As seen in Fig. 2b, stability of copper platform is high and comparable to the gold one.[26] The obtained results indicate great stability of prepared sample on the copper platform. The long term stability opens a new route to study analytes for which the long time preparation may affect the final results, particularly because of its non-constancy on a SERS platform. Additionally, these results proved that the observed Raman enhancing are due to both effects, the local field enhancement associated with resonant excitation of surface plasmons and chemical enhancement on the metal surface.

### The calculations of the enhancement factors and reproducibility of SERS platforms

All calculations of the enhancement factors were performed using the following formula:

$$EF = \frac{(I_{SERS} \times N_{NR})}{(I_{NR} \times N_{SERS})} \quad (1)$$

where $I_{SERS}$ is an SERS intensity of MGITC, $I_{NR}$ is a normal Raman scattering of MGITC, and the number of molecules are abbreviated as $N_{NR}$ and $N_{SERS}$. Laser spot diameter was calculated from the following formula:[30]

$$S_d = \frac{(1.22 \times \lambda)}{NA} \quad (2)$$

where λ denotes laser light wavelength and *NA* is the numerical aperture of an objective. With our setup, the $S_d$ was equal to $3.1 \times 10^{-6}$ m, and the illuminated area of the surface $S_0$ was $7.5 \times 10^{-12}$ m$^2$. Taking into account the area illuminated with the laser, the area of a platform ($5 \times 10^{-6}$ m$^2$), and the amount of molecules in a bulk, under these conditions, $2.25 \times 10^{13}$ MGITC molecules were irradiated through the laser light. As seen from both AFM (Fig. 1, Figure S2) and SEM images (Figure S3) and what is gathered in Table 1, the sizes of the copper crystals constituting the surface of a platform are in the range of 30–80 nm in diameter. Thus, to simplify the calculation, the smallest value of 30 nm copper diameter was taken into account, which is $1.5 \times 10^{-8}$ m for the laser radius. The following main requirements should be met for the previously mentioned calculations: (1) uniform distribution of the structures over the whole substrate surface associated with (2) regular and comparable linear dimensions, and (3) hemispheres as a shape of copper crystals formed on a platform. Presumably, the surface area for a single copper crystal is smaller than half of a sphere, which directly influences the less amount of MGITC, which may

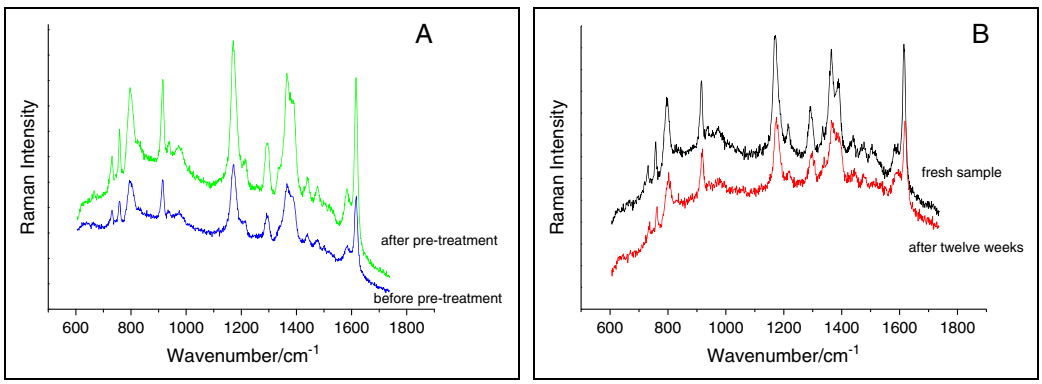


**Figure 2.** Surface enhanced Raman spectroscopy spectra of MGITC $10^{-6}$ M obtained for the following: (A) platforms prepared in two ways – MGITC is attached to the platform before and after treatment of acetic acid; and (B) stability of platform – spectra taken from the same sample at a different time. (This figure is available in colour online at wileyonlinelibrary.com/journal/jrs.)

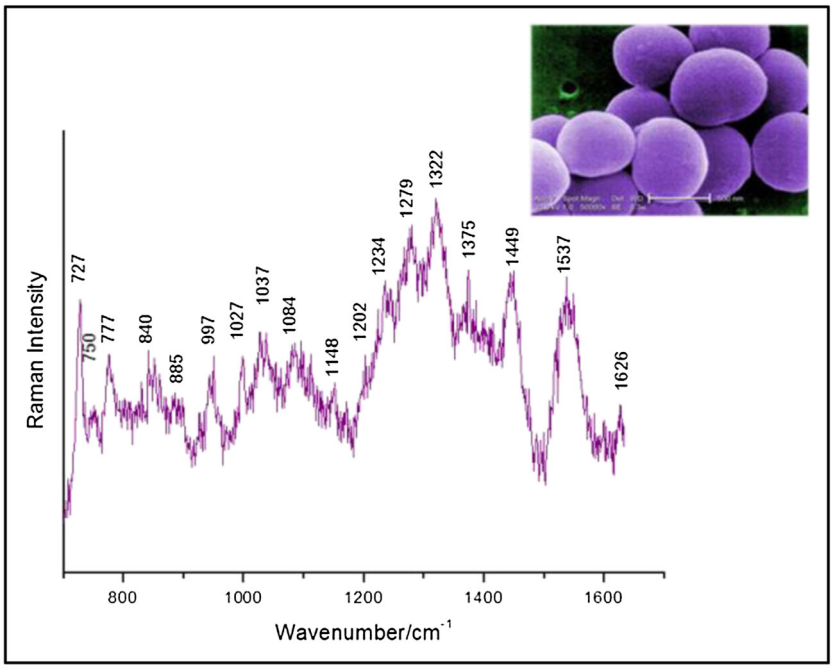


**Figure 3.** Surface enhanced Raman spectroscopy spectrum of *Staphylococcus aureus*. The inserted image of these bacteria is from Public Health Image Library. (This figure is available in colour online at wileyonlinelibrary.com/journal/jrs.)

attached to the platform surface, therefore, we consider the calculated factor to be underestimated. Consequently, the area of illumination for a single hemisphere ($S_{Sp}$), taken from the area of a disk, and the number of hemispheres ($N_{Sp}$) in the laser illumination can be calculated as follows:

$$S_{Sp} = \pi r^2 = 7.1 \times 10^{-16}\, m^2, N_{Sp} = {}^{S_0}/_{S_{Sp}} = 10563 \qquad (3)$$

This finally gives the total area of one hemisphere ($S_{TSp}$) as twice area of a disk:

$$S_{TSp} = 2\, S_{Sp} = 1.4 \times 10^{-15}\, m^2 \qquad (4)$$

Commonly, the malachite green molecules adapt a slightly inclined orientation (55°) at silica or mica surfaces.[31] Similarly, tilted adsorption geometry for MGITC was observed in our case, for a 55°-inclined MGITC molecule, the occupied surface area amounts to approximately $6 \times 10^{-19}\,m^2$.[26] If we assume all: (1) the maximum coverage of surface by MGITC molecules, (2) the surface of one molecule, and (3) the total area of one hemisphere, then the maximum coverage of one hemisphere is realized with 2333 MGITC molecules. Which number of MGITC, multiplied by $N_{Sp}$ (number of hemispheres), gives the number of MGITC probed molecules as $N_{SERS} = 2.46 \times 10^7$. All factors calculated for the studied platforms are presented in Table 1.

Moreover, the reproducibility of SERS spectra across a single platform and between different platforms was calculated using second derivative method (Supporting Information).[32] The obtained results (Fig. S4; Supporting Information) show high average spectral correlation coefficient $\Gamma = 0.82$. This result is comparable to the results of spectral correlation coefficients calculated for gold nanoparticles and commercial substrates (0.85).[33]

### Example of application – the detection of *Staphylococcus aureus* bacteria

The potential for our novel platforms to recognize bio-object has been determined with *S. aureus* as target bacteria. *S. aureus* is a bacterium frequently found in the human respiratory tract and on the skin. Commonly, it causes skin infections, respiratory disease, or food poisoning. Particularly, the antibiotic-resistant forms (e.g. MRSA) are considered to be a worldwide problem in clinical medicine. In order to demonstrate bacteria detection, a platform was prepared at 653 MPa (similar to the platform B), and untreated before applying the bacteria. Because of the high fluorescence of bacteria, the SERS spectra were taken for 785 nm wavelength. In Fig. 3 the spectrum of *S. aureus* bacteria presents their characteristic band vibrations. Thus, correspondingly, the band located at 727 $cm^{-1}$ is assigned to the C–N stretching mode of adenine part of lipid layer in the cell wall and/or to the purine ring breathing mode.[34,35] The same peak can also be assigned to the glycosidic ring mode from cell wall peptidoglycan.[36] We can also note spectral features at 840, 1027, 1037 and 1084 $cm^{-1}$. They come from tyrosine, phenylalanine, C–C oscillations, and from phosphate binding in DNA, respectively.[33] While analyzing SERS spectrum of *S. aureus*, it also was possible to observe other intense peaks assigned to amide III (1279 and 1322 $cm^{-1}$), $CH_2$ vibrations (1449 $cm^{-1}$), amide II (1537 $cm^{-1}$), and amide I (1626 $cm^{-1}$).[34,37] All observed bands were comparable with those observed when using silver or gold platforms with bacteria as analytes. This demonstrates that our novel platforms can enhance vibrations in biomolecules, which can have high potential for bioanalytical applications.

## Conclusion

Novel Cu-based SERS platforms have been prepared with a simple, reliable, and cost-effective high pressure method. The presented platforms are covered by uniformly distributed copper nanocrystals and give high enhancement factor up to $4.6 \times 10^7$. Noticeably, the recorded SERS spectra are reproducible both across a single platform and between different platforms. The average spectral correlation coefficient ($\Gamma$) was 0.82. The obtained platforms proved to be very stable over time, and reveal good enhancement of bacteria

bands, which confirms its potential for application in fundamental biological studies as well as in medical diagnostics.

**Acknowledgements**

The work of A. Kaminska was realized within the POMOST Program supported by the Foundation for Polish Science and co-financed by the EU 'European Regional Development Fund' POMOST/2010–2/10. A. A. Kowalska would like to thank Dr. Roman Luboradzki, and MSc Ewelina Magos for technical support. We also thank Dr. Tomasz Rolinski correlations of coefficients calculation and especially to Dr. Tomasz Szymborski for AFM measurements.

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